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ORIGINAL ARTICLE

Protoplast isolation and genetically true-to-type plant regeneration from leaf- and callus-derived protoplasts of *Albizia julibrissin*

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Abstract Protoplast isolation and subsequent plant regeneration of *Albizia julibrissin* was achieved from leaf and callus explants. Leaf tissue from 4 to 5-week-old in vitro seedlings was the best source for high-yield protoplast isolation. This approach produced 7.77×10^5 protoplasts (Pp) per gram fresh weight with 94% viability; after 60 min pre-plasmolysis with 0.7 M sorbitol followed by digestion in a solution of cell and protoplast wash plus 0.7 M mannitol, 1.5% cellulase Onozuka R10, and 1% pectolyase Y-23 for 6 h. Liquid Kao and Michayluk medium containing 2.7 µM α-naphthaleneacetic acid (NAA) and 2.2 µM 6-benzylaminopurine (BA) was best for sustained cell division and microcolony formation from both leaf- and callus-derived protoplasts at a density of $3–5\times10^5$ Pp ml^{-1}. Protoplast-derived microcalli became visible after 3–4 weeks on semi-solid medium of the same composition. Microcalli were then cultured on Murashige and Skoog (MS) medium containing Gamborg B5 vitamins or woody plant medium supplemented with different concentrations of NAA plus 4.4 µM BA for further growth. Proliferated leaf- and callus-protoplast-derived calli differentiated into microshoots on MS medium containing 13.2 µM BA plus 4.6 µM zeatin after 2–3 weeks, with an overall shoot organogenesis efficiency of 78–93%. Rooting of microshoots on half-strength MS medium containing 4.9 µM indole-3-butyric acid was successful, and plantlets were acclimatized to the greenhouse with a survival rate of >62%. Using ten start codon targeted and ten inter-simple sequence repeat primers, the genetic integrity of nine leaf- and six callus-protoplast-based plants was validated along with the mother seedlings.

Keywords *Albizia* · Cellulase · CPW · Genetic fidelity · Microcolony formation · Protoplast · Silk tree

Abbreviations

BA	6-Benzylaminopurine
CPW	Cell and protoplast wash solution
2,4-D	2,4-Dichlorophenoxyacetic acid
FDA	Fluorescein diacetate
IBA	Indole-3-butyric acid
ISSR	Inter-simple sequence repeats
KM	Kao and Michayluk medium
MS	Murashige and Skoog medium
MSB5	MS medium with Gamborg B5 vitamins
NAA	α-Naphthaleneacetic acid
PCR	Polymerase chain reaction
PGR	Plant growth regulator
Pp	Protoplast
Pp gfw^{-1}	Protoplasts per gram fresh weight
SCoT	Start codon targeted
TDZ	Thidiazuron
WPM	Woody plant medium

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Introduction

Albizia julibrissin Durazz (Fabaceae), commonly known as silk tree or mimosa, is a multipurpose tree legume native to Asia occurring from Iran to Japan, and is widely naturalized in many countries (Cheatham et al. 1995; Orwa et al. 2009). Leguminous woody plants, such as *A. julibrissin*, have become significant components of forest ecosystems, and have an ecological, economic, or medicinal role. Nitrogen fixation by *A. julibrissin* is beneficial to the forest, as well as improving soil fertility and land restoration (Jordan 2004; Pitman 2008). Silk tree seeds are a source of oil, the foliage can be used as fodder for livestock and other ruminants, and the species provides valuable cover for wildlife, while butterflies and honeybees consume nectar from the flowers (Wang et al. 2006; Meyer 2010; Nehdi 2011; Bouazza et al. 2012). Sladden et al. (1992) reported that plots of mimosa yielded approximately 10,087.7 kg ha^{-1} annual forage from four harvests per year. This species may therefore have potential use in improving traditional agroforestry systems. Because of its attractive flowers and canopy *A. julibrissin* has been planted as an ornamental, but the ornamental value of this species has been limited by sensitivity to *Fusarium oxysporium* f. sp. *perniciosum*, a soil-borne fungus that infects the root system causing vascular wilting and eventual death (Phipps and Stipes 1976). Various parts of *A. julibrissin* are of medicinal value and widely used in folk medicine. Flower heads of silk tree have been used in traditional medicine for the treatment of anxiety, depression, and insomnia (Kang et al. 2007). The triterpenoid juliberoside J_{28} from the bark of *A. julibrissin* was shown to inhibit the growth of three tumor cell lines in vitro (Liang et al. 2005). The tree also contains active natural products of additional pharmaceutical interest including flavonoids (Lau et al. 2007), phenolic glycosides (Jung et al. 2004), and triterpenoid saponins (Kinjo et al. 1992; Liang et al. 2005; Han et al. 2011). Two secondary metabolites present in the seed pods have the potential to act as a biocide and antioxidant (Lv et al. 2011).

Plant protoplasts provide an experimental single cell method for studies on a number of important fields in plant biotechnology, such as somatic hybridization by protoplast fusion, somaclonal (protoclonal) variation, genetic manipulation, functional characterization of plant genes, and genome editing (Davey et al. 2005b; Grosser et al. 2010). Advancement in the genetic improvement of some commercially important woody plants has been achieved by exploiting these protoplast-to-plant regeneration systems (Fu et al. 2011; Grosser and Gmitter 2011; Soriano et al. 2012; Xiao et al. 2014). Protoplasts have also been successfully utilized as a physiological information system in *Populus* (Guo et al. 2012; Tan et al. 2013) and a few other plant species, to study transient gene expression and to determine subcellular protein localization, as well as many other cellular characterizations, such as protein–protein and protein-DNA interactions (Yoo et al. 2007; Faraco et al. 2011). Although advancements in protoplast isolation, culture, and protoplast-to-plant regeneration have been considerable for herbaceous, agronomic, and fruit-tree species, the development of complete protocols for temperate tree species (excluding conifers) has been limited. Yellow-poplar (*Liriodendron tulipifera*) plantlets derived from somatic embryos, differentiated from embryogenic callus derived from protoplasts, has been reported (Merkle and Sommer 1987). Plantlet regeneration from protoplasts isolated from suspension cultures derived from seed-induced calli of a non-hybrid poplar (*Populus alba*) was successful (Qiao et al. 1998). Camphor tree (*Cinnamomum camphora*) plantlets were regenerated from protoplasts isolated from embryogenic suspension cultured cells (Du and Bao 2005). Leaf mesophyll-derived protoplasts of mulberry (*Morus indica*) were regenerated into shoots and a few plants were established in the greenhouse (Umate et al. 2005). Kanwar et al. (2009) developed a protocol for plant regeneration from callus- and mesophyll-derived protoplasts of the leguminous black locust (*Robinia pseudoacacia*). The successful regeneration of plants from American elm (*Ulmus americana*) cell suspension-derived protoplasts has recently been reported (Jones et al. 2015).

Previous investigations on in vitro culture of *A. julibrissin* have centered on adventitious shoot regeneration (Sankhla et al. 1993, 1994, 1995, 1996; Zhou et al. 2001; Rahmani et al. 2015) and somatic embryogenesis (Burns and Wetzstein 1998), but there have been no reports on protoplast isolation, culture, and plant regeneration of this species. Therefore, the present study was initiated to develop a method for protoplast isolation, microcalli formation, and subsequent shoot regeneration and rooting of *A. julibrissin* that could then be the foundation for genetic studies. Inter-simple sequence repeats (ISSR) and start codon targeted (SCoT) polymorphism were used to assess the genetic fidelity or variability of in vitro-regenerated plants from protoplasts of *A. julibrissin*.

Materials and methods

Plant material

Seeds of *A. julibrissin* were excised from mature pods collected from a 40- to 50-year-old tree growing in the Sisangan area of the Hyrcanian forest, northern Iran. Seeds were rinsed under running tap water for 20 min, immersed in 85–90 °C water (Fordham 1965) that was then allowed to gradually cool to room temperature. Treated seeds were then surface disinfested by soaking in 70 % (v/v) ethanol for 3 min, followed by immersion in 20 % (v/v) bleach solution (5.25 %

sodium hypochlorite) containing three drops of Tween 20 per 100 ml for 3 min, then rinsed three times (3 min each) in sterile distilled water. Clean seeds were air dried on sterile tissue and were then cultured on plant growth regulator-free Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 30 g l^{-1} sucrose and 7 g l^{-1} plant agar (Duchefa, Biochemie BV, Netherlands) for germination; which usually occurred within 6–10 days at 26 ± 2 °C under a 16 h photoperiod (40 µmol m^{-2} s^{-1}).

Expanded leaves from the apex of 4–5-week-old in vitro-germinated seedlings were used as explants for protoplast isolation. For protoplast isolation from callus, additional seeds were cultured on the same medium for germination but were kept in the dark for 10–14 days to obtain elongated and etiolated hypocotyls. Hypocotyl segments (5–7 mm) were excised and cultured horizontally on MS medium containing 0.2 g l^{-1}*myo*-inositol (Duchefa) and supplemented with 10.8 µM α-naphthaleneacetic acid (NAA) and 4.4 µM 6-benzylaminopurine (BA) for induction of white, friable, organogenic callus (Rahmani et al. 2015).

Protoplast isolation

The enzyme solution was a mixture of different concentrations and combinations of cellulase Onozuka R-10 and either macerozyme R-10 or pectolyase Y-23 (Duchefa; Tables 1, 2). Enzymes were dissolved in cell and protoplast washing (CPW) salt solution (Frearson et al. 1973) containing 0.7 M [13 % (w/v)] mannitol as osmoticum (CPW13M), and buffered with 5 µM 2-(N-morpholino)ethanesulfonic acid (MES). The pH of the solution was adjusted to 5.5–5.8 and then filter-sterilized through a 0.45 µm filter disc (Millipore, Billerica, MA, USA). Solutions were stored at −20 °C in 5 ml aliquots until used. To inactive protease enzymes, the enzymatic solution was heated at 55 °C for 10 min prior to tissue digestion treatment.

Protoplasts were isolated separately from two types of plant tissue, leaves from 4 to 5-week-old in vitro-seedlings and hypocotyl-derived organogenic callus. Thin strips of leaves (1 g) or callus (1 g) were incubated in sterile 10 cm Petri dishes containing 20 ml CPW solution with 0.7 M mannitol or sorbitol pre-plasmolysis solution for 30, 60, or 90 min in the dark at 25 ± 2 °C. Each type of tissue (250 mg) was then transferred to a sterile 50-ml Erlenmeyer flask containing 5 ml enzyme solution. Incubation in the enzyme solution was carried out in darkness for different durations (in vitro leaf strips: 3, 6, 9, or 12 h; callus: 8, 12, 16, or 20 h) at 25 ± 2 °C with gentle shaking (40 rpm). Following incubation, the protoplast-enzyme mixture was diluted by adding an equal volume of CPW13M. The protoplast-enzyme solution was then filtered through 0.75 µm nylon mesh (Millipore) and the nylon mesh was rinsed with CPW13M. The filtrate was transferred to 15 ml sterile plastic centrifuge tubes, centrifuged at 100 × g for 5 min, followed by discarding the supernatant without disturbing the protoplast pellet. The protoplast pellet was then washed with 15 ml CPW13M solution, vortexed,

Table 1 Effect of concentration and combination of enzymes in digestion solution and digestion period on protoplast yield and viability from in vitro leaves of *Albizia julbrissin*

Enzymes (%)	Protoplast yield (×10^5 Pp gfw^{-1}) and viability (%) after digestion period (h)									
	3		6		9		12		Average	
	Y	V	Y	V	Y	V	Y	V	Y	V
1.0 C + 0.3 M	0.03u	95	2.26t	86	2.47 s	63	3.35p	62	2.02	77
1.0 C + 0.5 M	0.05u	93	2.64r	86	3.22p	65	3.53o	63	2.36	77
1.5 C + 0.3 M	0.05u	92	2.87q	88	3.78n	64	4.07m	60	2.69	76
1.5 C + 0.5 M	0.06u	87	3.26p	86	4.29l	62	4.36k	60	2.99	74
2.0 C + 0.3 M	0.06u	88	4.20l	85	4.78i	59	5.44g	52	3.62	71
2.0 C + 0.5 M	0.08u	92	4.58j	81	5.07h	54	5.88d	50	3.9	69
1.0 C + 0.5 P	0.12u	93	4.74i	82	5.26h	58	5.57e	58	3.92	73
1.0 C + 1.0 P	0.12u	92	5.54e	83	6.07c	57	6.89a	50	4.66	70
1.5 C + 0.5 P	0.15u	91	5.61e	81	5.92d	53	6.19c	52	4.47	69
1.5 C + 1.0 P	0.25u	92	6.31c	87	6.38b	51	6.72b	48	4.92	67
2.0 C + 0.5 P	0.20u	90	5.40g	79	6.03c	54	6.37b	52	4.5	68
2.0 C + 1.0 P	0.21u	87	5.41g	81	6.24c	55	6.78a	49	4.66	68
Average	0.11	91	4.4	83	4.95	57.9	5.42	54	3.73	71.58

Means with the same letter within columns are not significantly different at $P = 0.05$ according to LSD test

C cellulase onozuka R-10, *M* macerozyme R-10, *P* pectolyase Y-23, *Pp* gfw^{-1} protoplasts per gram fresh weight of donor tissue, *V* viability, *Y* yield

Table 2 Effect of concentration and combination of enzymes in digestion solution and digestion period on protoplast yield and viability from hypocotyl callus of *Albizia julbrissin*

Enzymes (%)	Protoplast yield ($\times 10^5$ Pp gfw^{-1}) and viability (%) after digestion period (h)									
	8		12		16		20		Average	
	Y	V	Y	V	Y	V	Y	V	Y	V
1.0 C + 0.3 M	0.02r	99	0.06r	85	2.06m	82	4.07h	56	1.55	81
1.0 C + 0.5 M	0.01r	97	0.31q	85	2.52k	89	4.20h	50	1.76	78
2.0 C + 0.3 M	0.01r	97	0.31q	86	2.14l	92	4.16h	53	1.66	80
2.0 C + 0.5 M	0.01r	97	1.18o	86	3.08j	83	4.25h	52	2.13	80
3.0 C + 0.3 M	0.05r	95	1.26n	85	3.23i	88	5.16e	52	2.43	79
3.0 C + 0.5 M	0.02r	94	1.35n	87	3.44i	84	5.00f	51	2.45	79
1.0 C + 0.5 P	0.08r	93	1.51n	86	3.22i	82	5.43c	53	2.56	79
1.0 C + 1.0 P	0.09r	94	1.46n	94	4.31g	83	5.29d	56	2.79	81
2.0 C + 0.5 P	0.08r	90	1.11o	92	4.07h	87	6.25c	51	2.88	78
2.0 C + 1.0 P	0.15q	95	1.81m	89	5.53c	85	6.63a	50	3.53	79
3.0 C + 0.5 P	0.12q	93	1.07p	89	4.53g	82	6.25b	52	2.99	79
3.0 C + 1.0 P	0.14q	94	1.07p	91	4.10h	83	5.46c	47	2.69	79
Average	0.07	95	1.04	88	3.52	85	5.18	52	2.45	79.33

Means with the same letter within columns are not significantly different at $P = 0.05$ according to LSD test

C cellulase onozuka R-10, *M* macerozyme R-10, *P* pectolyase Y-23, *Pp gfw*$^{-1}$ protoplasts per gram fresh weight of donor tissue, *V* viability, *Y* yield

and centrifuged at 100×*g* for 3 min. Protoplasts were then re-suspended in 3 ml Kao and Michayluk (KM; 1975) plant growth regulator-free liquid medium prior to yield and viability assessment. Protoplast yield (number of protoplasts per gram of tissue) was determined by using a hemocytometer, and counting the number of protoplasts in 50 µl protoplast suspension. Protoplasts were stained with 0.05 % (w/v) fluorescein diacetate (FDA; Sigma) to assess viability (Widholm 1972). A solution of 0.05 % FDA was prepared in CPW13M solution and 100 µl freshly isolated protoplast suspension was placed in 400 µl stain solution for 2–5 min. Viability rate of freshly isolated protoplasts was assessed under ultraviolet light, and expressed as the ratio of the number of viable protoplasts to the total number of protoplasts (%). Experiments were carried out three times, and the average number of total protoplasts and viable protoplasts were recorded.

Protoplast culture

Isolated protoplasts were rinsed with 10 ml CPW13M solution and then maintained for 30 min at room temperature to settle. Two different culture protocols were utilized for culture of *A. julibrissin* leaf- or callus-protoplasts: liquid culture and agarose-embedded culture.

For liquid culture, the rinse solution was replaced with either B5 (Gamborg et al. 1968), MS (minus NH_4NO_3), or KM8p (Kao and Michayluk 1975; without the nucleic acid bases) basal salt medium and vitamins, supplemented with 0.5 g l^{-1} casein hydrolysate, 2 % (w/v) sucrose, 1 % (w/v) glucose, 0.7 M mannitol, and 2.7 or 5.4 µM NAA or 2.3 or 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 2.2 µM BA. The purified protoplasts were cultured in 9 cm (diameter) glass Petri dishes containing 6 ml liquid medium with a plating density of 3–3.5 $\times 10^5$ Pp ml^{-1}. Prior to culture, the pH of the medium was adjusted to 5.8 using 1 N NaOH followed by autoclaving at 121 °C for 20 min. Culture dishes were placed in the dark at 25 ± 2 °C without agitation. The osmotic strength of the culture medium was lowered by adding 500 µl fresh medium without mannitol to the culture dishes at 4 days intervals. For agarose-embedded culture, KM8p basal medium (pH 5.8) containing either 2.7 or 5.4 µM NAA or 2.3 or 4.5 µM 2,4-D in combination with 2.2 µM BA, 0.2 % (w/v) MES, and 1.4 % (w/v) SeaPlaque™ agarose (Duchefa) was filter-sterilized and maintained at 45 °C. Aliquots of protoplast suspension with a final density of 3.5 $\times 10^5$ Pp ml^{-1} were mixed carefully at a ratio of 1:1 with the agarose medium (final agarose concentration 0.7 %) at room temperature. After dispensing (2 ml) and solidifying (60 min incubation at room temperature) the mixture in 60 mm × 12 mm Petri dishes, 5 ml liquid KM8p of the same composition plus 10 ml osmoticum solution (8 % (w/v) sucrose; pH 5.8; filter-sterilized) was added into each Petri dish, followed by gentle swirling to form a thin layer. This medium refreshing step was repeated when browning was just starting to be observed in the developing microcolony formation. The osmoticum solution was

also refreshed at 10 days intervals. The culture plates were sealed with Parafilm and incubated at 25 ± 2 °C in the dark with agitation (30–40 rpm).

Cultures were observed regularly for cell division and microcolony formation for 2 months after initial culture. The protoplast-derived microcolonies and cell clusters inside the agarose beads were gradually released into the liquid phase of the cultures. After browning the liquid phase and microscopic confirmation of microcolonies, half of the liquid phase of culture medium was replaced with a fresh equal volume. The liquid medium volume that was removed was transferred to a 5 ml tube followed by centrifugation (40×*g*). After removal of the supernatant, the pelleted microcolonies were washed with liquid KM8p culture medium and re-suspended in 1 ml KM8p liquid medium, and spread on the surface of freshly prepared 0.8 % agar medium supplemented with the same components used for the first culture, except that mannitol was eliminated, and cultures were in 100 mm × 12 mm Petri dishes for microcalli formation under 6 h photoperiod (6–8 µmol m^{-2} s^{-1}). The number of microcolonies and microcalli formed per petri dish was recorded.

Callus formation and shoot regeneration

After 3 weeks, microcalli (2–4 mm in diameter) were transferred to petri dishes containing MSB5 medium (MS medium with Gamborg B5 organics) or woody plant medium (WPM; Lloyd and McCown 1981) containing 3 % sucrose, 0.8 % agar (Duchefa), 5.4, 8.1, or 10.8 µM NAA, 4.4 µM BA, and 0.2 g l^{-1} casein hydrolysate for further growth and proliferation. Callus was sub-cultured to fresh treatment medium every 2 weeks. Shoot organogenesis (MS medium with 13.2 µM BA and 4.6 µM zeatin), in vitro rooting (half-strength MS medium with 4.9 µM indole-3-butyric acid; IBA), and acclimatization of regenerated plants to ex vitro conditions were achieved following our previously established protocol (Rahmani et al. 2015).

Statistical data analysis

All treatments were replicated three times and three independent experiments were performed for each treatment. The data were subjected to statistical one-way analysis of variance (ANOVA) using SAS 9.1 (SAS Institute 2004). The mean value and standard error of all treatments were calculated. When the ANOVA of treatment means was statistically significant, Fisher's least significant difference (LSD) test was used to distinguish differences between treatments ($p = 0.05$).

DNA isolation and genetic fidelity screening

Genetic identity of in vitro-germinated seedlings was checked with ISSR primers prior to establishment as source material. Plants with a genetically homogeneous background (Supplementary Fig. 1) were then selected and used as the original source material. The DNA of these selected open-pollinated seedlings was used as control seedlings in genetic fidelity screening of the protoplast-derived plants.

Developing leaf material from in vitro mother seedlings and 15 regenerated plants from protoplasts (nine leaf-protoplast based and six callus-protoplast based) were collected (50–100 mg) and ground for genomic DNA extraction using the cetyl trimethyl ammonium bromide protocol of Doyle and Doyle (1990). DNA quality was checked using visual comparative loading in ethidium bromide-stained 0.8 % (w/v) agarose gel with a known concentration of 100 bp DNA ladder (SinaClon, Karaj, Iran). The quantity of isolated DNA was checked using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany), and the concentration of DNA samples was adjusted to 20–25 ng μl^{-1} final concentration using TE buffer, and stored at −20 °C until used.

Among the 20 SCoT and 15 ISSR primer sequences screened, ten and eight primers (Supplementary Table 1), respectively, produced clear and reproducible bands, and were selected for genomic DNA amplification to show possible genetic variability of plants. Polymerase chain reaction (PCR) was performed in a 25 µl reaction mixture containing 12.5 µl 2× Master-Mix buffer (0.08 U μl^{-1} Taq polymerase, 3 mM $MgCl_2$, 0.4 mM of each dNTP) (SinaClon), 0.8 µM of each SCoT or ISSR primer, 40–50 ng genomic DNA, and sterile nuclease-free distilled water. The PCR amplifications were carried out in a Bio-Rad C-1000 thermal cycler with an initial denaturation for 5 min at 94 °C, then 36 cycles of 60, 60, and 120 s at 94 °C, 50 °C (for SCoT primers) or 45–60 °C (for ISSR primers), and 72 °C, respectively, with a final extension at 72 °C for 10 min; then stored at 4 °C. All PCR reaction mixtures were overlaid with 8 µl mineral oil. In order to visualize possible self-polymerization or DNA contamination, a negative control PCR reaction with sterile distilled water in place of genomic DNA was added to the PCR series. The stability of PCR conditions was checked using a positive PCR reaction with a known DNA template and the specific forward and reverse primers producing a 620 bp product. Two independent PCR reactions were conducted for all amplifications reactions.

Amplified PCR products were visualized and photographed under ultraviolet light (Bio-Rad Molecular Imager XR^+ system).after electrophoresis [1.2 % agarose (w/v; SinaClon) plus 1 µg ml^{-1} ethidium bromide; Merck, Germany]. A 1-kb (for SCoTs) or 100-bp (for ISSRs) DNA ladder (SinaClon) was used as molecular weight ruler.

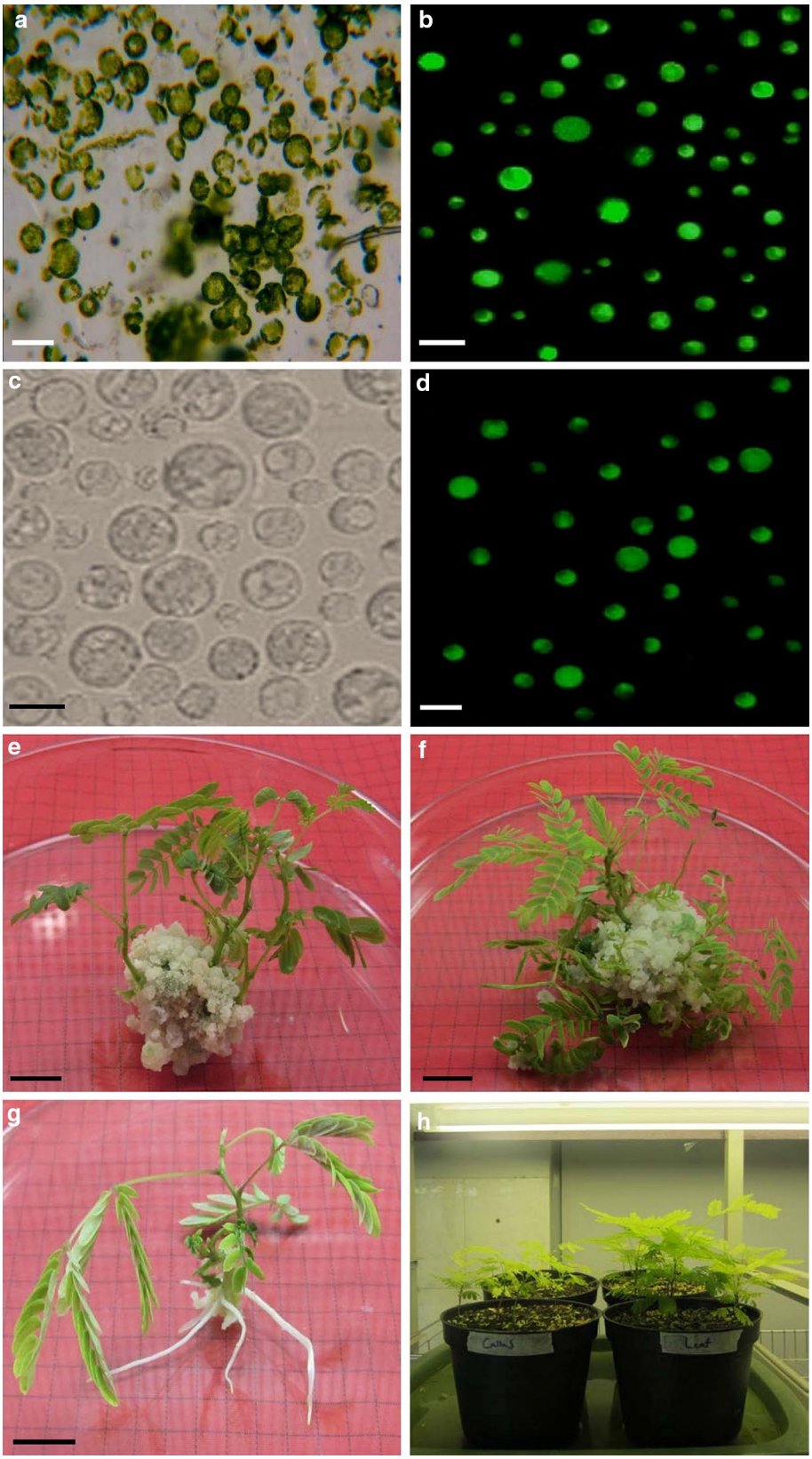

Fig. 1 Protoplast isolation and plant regeneration from leaf- and callus-derived protoplasts of *Albizia julibrissin*. **a, b** Freshly isolated purified protoplasts from in vitro grown leaves and stained with FDA. **c, d** Freshly isolated purified protoplasts from callus and stained with FDA. **e, f** Leaf- and callus-protoplast regenerated adventitious shoots on MS medium with 13.2 μM BA plus 4.6 μM zeatin after 5 weeks. **g** Rooted shoot regenerated from leaf-derived protoplast; **h** Acclimatized plants from leaf- (*right*) and callus- (*left*) derived protoplasts (*Bars* 1 cm)

Table 3 Effect of osmoticum and pre-plasmolysis time on yield and viability of *Albizia julbrissin* protoplasts

Donor tissue	Osmoticum (0.7 M)	Time (min)	Response	
			Yield $\times 10^5$ Pp gfw^{-1}	Viability (%)
In vitro leaves (digestion in 1.5 % C + 1 % P after 6 h incubation)	Mannitol	Control	6.31 ± 0.1cd	87 ± 2.0c
		30	6.46 ± 0.0c	82 ± 3.7bc
		60	7.07 ± 0.0b	81 ± 4.3bc
		90	6.17 ± 0.2cd	86 ± 2.1ab
	Sorbitol	30	7.01 ± 0.0b	92 ± 2.5a
		60	7.77 ± 0.0a	94 ± 2.6a
		90	6.05 ± 0.0d	92 ± 1.7a
Callus (digestion in 2 % C + 1 % P after 16 h incubation)	Mannitol	Control	5.53 ± 0.1ef	85 ± 2.6bc
		30	5.50 ± 0.0f	80 ± 5.7c
		60	5.80 ± 0.0d	84 ± 2.8abc
		90	5.76 ± 0.0de	82 ± 3.1abc
	Sorbitol	30	6.17 ± 0.0c	91 ± 3.2ab
		60	6.53 ± 0.0b	90 ± 2.8abc
		90	6.92 ± 0.0a	92 ± 2.0a

Values represent means ± SE for three replications in each treatment. Means with the same letter within columns are not significantly different at $P = 0.05$ according to LSD test

C cellulase onozuka R-10, *P* pectolyase Y-23

Results

Protoplast isolation from leaves and callus

Protoplasts were successfully isolated via enzymatic digestion from in vitro leaves and callus of silk tree (Fig. 1a, c, respectively), and were stained with FDA (Fig. 1) to determine viability. The yield and viability of protoplasts were significantly different depending on the starting tissue and duration of tissue incubation for digestion (Tables 1, 2). There were also significant differences in protoplast yield and viability between enzyme solutions. Protoplast yield significantly increased with increasing enzyme concentration, but mean viability decreased when concentration of cellulase and macerozyme or pectolyase were increased to 2, 0.5, and 1 %, respectively. With leaf strips, protoplast yield varied between 0.03 and 6.89×10^5 Pp gfw^{-1} in the presence of different concentrations of cellulase and macerozyme or pectolyase (Table 1), while the protoplast yield from callus was 0.01–6.63×10^5 Pp gfw^{-1}. Digestion of leaf strips in 1.5 % cellulase and 1 % pectolyase for 6 h gave the best healthy protoplast yield as it released 6.31×10^5 Pp gfw^{-1} with 87 % viability (Table 1). The best yield of protoplasts from callus (5.53×10^5 Pp gfw^{-1} with 85 % viability) was obtained with 2 % cellulase plus 1 % pectolyase for 16 h (Table 2). Between the two tissues, leaf strips yielded on average more protoplasts (3.73 Pp gfw^{-1} with 71.6 % viability) than callus (2.45 Pp gfw^{-1} with 79.3 % viability). Macerozyme or pectolyase were compared to evaluate efficiency of healthy protoplast isolation in combination with cellulase. The inclusion of pectolyase in lieu of macerozyme enhanced the yield of protoplasts in all starting materials. The extension of digestion period increased the protoplast yield from both tissues. However, the mean viability of isolated protoplasts decreased to 54 % and 52 % after 12 h digestion of leaves and 20 h of callus, respectively. Donor tissues were pre-plasmolyzed in CPW with either 0.7 M mannitol or sorbitol for 30, 60, or 90 min. significant differences in protoplast yield and viability were observed among osmotica and pre-plasmolysis durations. Sixty-min pre-plasmolysis of in vitro leaves and 90 min pre-plasmolysis of callus in 0.7 M sorbitol was the most efficient pretreatment for further achievement of protoplasts (Table 3).

Protoplast culture and microcolony formation

Purified protoplasts derived from in vitro leaves and calli were cultured separately at a plating density of 3–3.5×10^5 Pp ml^{-1} for cell wall regeneration, cell multiplication, and microcolony formation. Leaf- and calli-derived protoplasts cultured in both liquid and agarose systems were able to synthesis cell walls, divide, and form cell colonies. Cells became larger and oval in shape within 30–48 h after culture in medium augmented with auxins (NAA or 2,4-D) plus BA, showing new cell wall synthesis followed by first cell division. Further divisions of the leaf- and callus- derived protoplasts led to the formation of several daughter cells and eventually microcolony development.

Three modified basal media (B5, MS, and KM8p) as liquid or semi-solid medium, and NAA or 2,4-D plus 2.2 µM

BA, were assessed for the effect on microcolony formation and subsequent microcalli growth (Supplementary Table 2). In addition, KM8p medium supplemented with 1.4% agarose was compared. In liquid culture, KM8p medium supplemented with 2.7 or 5.4 µM NAA plus 2.2 µM BA were the most effective media for the highest microcolony formation from leaf- and callus-derived protoplasts (Supplementary Table 2) after 3 weeks of culture (52.3 microcolonies for leaf-protoplasts, and 49.3 and 46.0 microcolonies for callus-protoplasts per Petri plate, respectively). When leaf- and callus-derived protoplasts were cultured in MS liquid medium containing 4.5 µM 2,4-D plus 2.2 µM BA, however, microcolony formation was reduced to ten and eight, respectively. The KM8p agarose embedded culture medium containing 2.7 µM NAA plus 2.2 µM BA gave the maximum response of 47 microcolonies (leaf) and 25 microcolonies (callus) as compared to the other PGRs tested of this culture system (Supplementary Table 2).

Callus formation and plant regeneration

After 3 weeks, growth of the protoplast derived microcolonies on semi-solid medium containing the same components of those used for the first culture (except that mannitol was eliminated), resulted in the initiation of microcalli 2–5 mm in diameter. KM8p medium supplemented with 2.7 or 5.4 µM NAA plus 2.2 µM BA resulted in the highest number of microcallus formation from microcolonies (Supplementary Table 2). The callus formed from both types of protoplast-derived microcolonies was transferred to semi-solid MSB5 medium (MS minerals with Gamborg B5 organics) or WPM containing different concentrations of NAA in combination with 4.4 µM BA (Supplementary Table 3) for further growth and proliferation. MSB5 medium with 10.8 µM NAA plus 4.4 µM BA was best for proliferation of microcalli from both leaf- and callus-derived protoplasts (Supplementary Table 3). In addition, MSB5 medium supplemented with 8.1 µM NAA plus 4.4 µM BA, and WPM with 10.8 µM NAA plus 4.4 µM BA were also suitable media for proliferation of microcalli from callus-derived protoplasts. WPM with 5.4 µM NAA plus 4.4 µM BA produced the least amount of callus proliferation from the leaf (35%) and callus (27.5%) protoplast-derived microcalli after 4 weeks of culture.

For shoot organogenesis, leaf- and callus-based protoplast derived calli were separately cultured on MS medium containing 13.2 µM BA plus 4.6 µM zeatin according to our previous protocol (Rahmani et al. 2015). Microshoots started to regenerate after 2–3 weeks culture. The overall shoot organogenesis efficiency of this culture medium was 78–93% (data not shown). Number of shoots from a single callus cluster varied from two to four (Fig. 1) after 5 weeks culture. Following our previous protocol (Rahmani et al. 2015), in vitro shoots were successfully rooted in half-strength MS medium supplemented with 4.9 µM IBA. On average, 68 and 61% adventitious root formation was observed for leaf- and callus-protoplast-derived shoots within 4–5 weeks culture, respectively (Fig. 1). Rooted shoots (Fig. 1) were then acclimatized, with over 60% of the plants showing normal growth and development after 5 weeks after transplanting to soil.

Genetic fidelity screening

Two DNA-based molecular markers, SCoT and ISSR, were applied to demonstrate genetic homogeneity of a total of nine and six regenerated plants from leaf- and callus-derived protoplasts, respectively. Initially 20 SCoT and 15 ISSR primers were screened, and ten and eight primers, respectively, amplified clear and scoreable bands. A total of 53 monomorphic bands were produced from PCR amplification of SCoT primers with amplicons ranging in size from 200 to 1300 bp, giving rise to monomorphic patterns across nine leaf- and six callus-protoplast-derived plants (Supplementary Table 1). The number of scoreable bands from each SCoT primer ranged from four (SCoT-5, SCoT-25, and SCoT-28) to eight (SCoT-24) with an average of 5.3 bands per primer (Supplementary Table 1). All ISSR primers yielded 55 scoreable and reproducible bands ranging from 11 (maximum) to five (minimum) bands from primers UBC-873 and UBC-811, 841, respectively (Supplementary Table 1), with an average of 6.8 bands per primer ranging in size from 300 to 1700 bp.

The amplification profiles were found monomorphic across all of the genotypes regenerated from both leaf- and callus-derived protoplasts by all SCoT and ISSR primers used. This finding showed the true-to-type nature of the genetic background of the protoplast-derived in vitro regenerated plants of *A. julibrissin*. The monomorphic banding of SCoT-1 and UBC-834 primers representative gels from genotyping nine leaf-protoplast-based regenerated genotypes and the mother seedling are shown in Fig. 2. The monomorphic fingerprinting profiles of the six callus-protoplast-derived in vitro plants and the mother seedling using SCoT-24 and UBC-873 are shown in Fig. 3.

Discussion

Since genetically altered organisms are not generally accepted by society, there is an interest in using protoplast-to-plant advancements in the generation of novel germplasm for plant improvement (Eeckhaut et al. 2013). One of the most recognized protoplast-based technologies utilizes protoplast fusion to produce unique plants with new or improved desirable traits. Successful culture and genetic

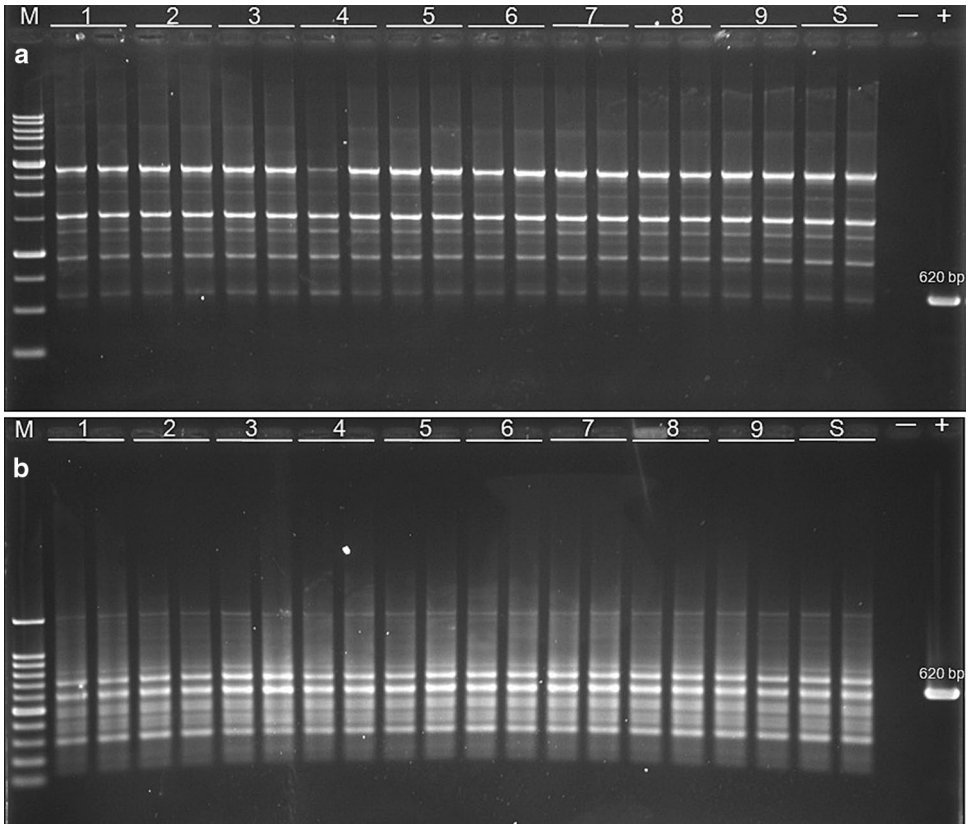

Fig. 2 Genetic fingerprinting of leaf-derived protoplast regenerated plants (*Lanes 1–9*) and mother seedlings (S). **a** SCoT banding of amplification products with primer SCoT-1 (M, 1 kb molecular marker), **b** ISSR banding of amplification products with primer UBC-834. (M, 100 bp molecular marker)

transformation of purified protoplasts depends on cell wall re-synthesis and subsequent plant regeneration (Davey et al. 2005b). Establishment of a protoplast-to-plant regeneration framework is affected by many components, including the protoplast isolation process, culture density and system, and medium composition (Eeckhaut et al. 2009; Kanwar et al. 2009; Castelblanque et al. 2010; Rezazadeh and Niedz 2015). As the regenerative capacity of protoplasts from different genotypes and tissues varies significantly, such investigations are required (Davey et al. 2005a, b). To our knowledge, this is the first study to investigate protoplast isolation and regeneration capacity of leaf- and callus-derived protoplasts in *Albizia julibrissin*.

Isolation of a large number of healthy protoplasts is a prerequisite for successful protoplast culture and regeneration. Several factors including protoplast donor tissue, pretreatment of tissue before enzyme maceration, pH and composition of enzyme solution, temperature and duration of enzyme incubation, gentle agitation, and protoplast isolation method (Frearson et al. 1973; Rao and Prakash 1995; Ortin-Parraga and Burgos 2003; Sinha et al. 2003; Kanwar et al. 2009; Kiełkowska and Adamus 2012) significantly affected the yield and viability of protoplast isolation. Therefore, to determine a method for silk tree protoplast isolation, a number of parameters including source material, enzymatic solution composition, digestion duration, and plasmolysis treatment were assessed. In the present investigation, leaves from in vitro grown seedlings and hypocotyl-derived organogenic callus were used as explant material for protoplast isolation. Although protoplasts can be isolated from different types of explants, it was reported that only young leaves of in vitro grown shoots, and actively growing callus or cell suspension cultures gave consistent and high yields of healthy protoplasts in woody plants (Liu 2005).

Large numbers of purified viable protoplasts (10^5–10^7 Pp gfw^{-1}) are required for protoplast fusion or *Agrobacterium*-mediated genetic modification (Davey et al. 2005a). The yields of protoplasts released from tissues in our study, varied from 0.03 to 7.77×10^5 Pp gfw^{-1} for leaves and 0.01–6.92×10^5 Pp gfw^{-1} for calli slices. Suitability of in vitro leaves for protoplast isolation in other woody plant species has been documented by Rezazadeh and Niedz (2015) in guava (*Psidium guajava*) (Yield 3.7×10^6; Viability > 90 %), Kanwar et al. (2009) in black locust (Yield 9.45×10^5; Viability 94.1 %), and Conde and Santos (2006) in European field elm (*Ulmus minor*) (Yield 3.96×10^7; Viability

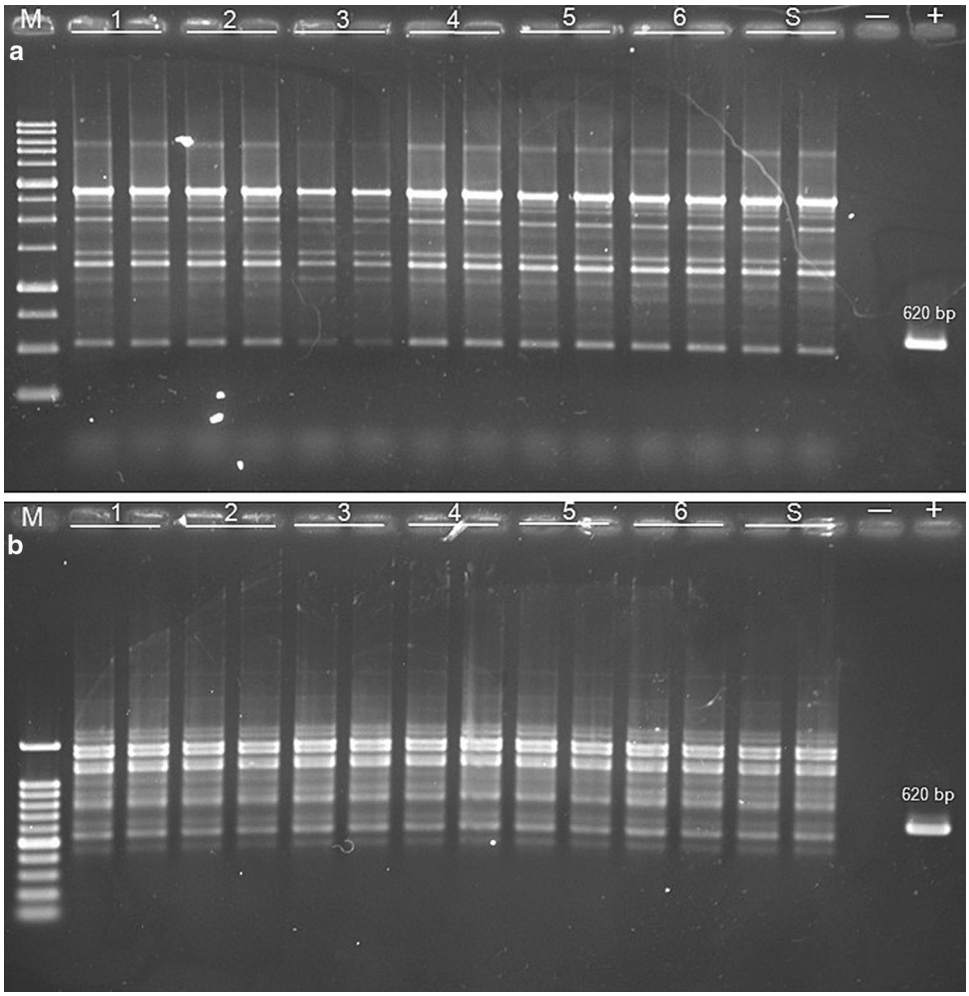

Fig. 3 Genetic fingerprinting of callus-derived protoplast regenerated plants (*Lanes 1–6*) and mother seedlings (S). **a** SCoT banding of amplification products with primer SCoT-24 (M, 1 kb molecular marker), **b** ISSR banding of amplification products with primer UBC-873. (M, 100 bp molecular marker)

60–80 %). However, compared to the in vitro leaf explants (Avg. Yield 3.73×10^5; Avg. Viability 71.6 %), slightly more viable protoplasts (Avg. Yield 2.45×10^5; Avg. Viability 79.3 %) were released from callus cells (Tables 1, 2). The difference between the protoplast yields of different tissues could be a result of the physiological state of the cells, such as structural and chemical differences in cell wall composition (Davey et al. 2005b). Similar differences were observed by Conde and Santos (2006) in European field elm, where, compared to calli slices and greenhouse leaves, digestion of in vitro leaves resulted in a significant increase in protoplast yield.

In addition to source tissue, results showed that incubation time, type, and proportion of enzymes in the digestion solution were determining factors in protoplast yield and viability in silk tree. Incubation in solutions containing 2 % cellulase plus 0.5 % macerozyme for 6 h (leaves; Yield 4.58×10^5 Pp gfw^{-1}) and 3 % cellulase plus 0.5 % macerozyme for 16 h (callus; Yield 3.44×10^5 Pp gfw^{-1}) produced maximum yields with 81 % and 84 % viability, respectively (Tables 1, 2). Kanwar et al. (2009) using black locust, reported that 2 % cellulase and 0.3 % macerozyme for 20 h (leaf-mesophyll; Yield 9.45×10^5) and 2 % cellulase plus 1 % macerozyme produced maximum yields with 94.1 % and 95.5 % viability, respectively. The combination of 1 % cellulase and 0.25 % macerozyme for 16 h (leaves; Yield 7.04×10^6) and 1 % cellulase plus 0.25 % macerozyme (callus; Yield 2.90×10^5) was reported best for enzyme digestion and healthy protoplasts from European field elm (Conde and Santos 2006). In silk tree, the viability of protoplasts ranged from 48 to 95 % (Avg. 71.6 %) for leaf tissue and 47–99 % (Avg. 79.3 %) in calli explants over all enzyme combinations tested. The inclusion of pectolyase in lieu of macerozyme enhanced the yield of protoplasts in silk tree (Tables 1, 2). Incubation in solutions containing 1.5 % cellulase plus 1 % pectolyase for 6 h (leaves; Yield 6.31×10^5

Pp gfw^{-1}) and 2% cellulase plus 1% pectolyase for 16 h (callus; Yield 5.53×10^5 Pp gfw^{-1}) produced maximum yields with 87% and 85% viability, respectively (Tables 1, 2). Similarly, Dorion et al. (1994) reported in *Ulmus* sp. (elm) that pectolyase showed a high performance in the isolation of protoplasts from in vitro leaves. When suspension cultures of other hardwood tree species were used for protoplast isolation, the type, concentration, and duration of incubation varied considerably; i.e. yellow-poplar: 2% cellulysin and 1% macerase, 12 h (Merkle and Sommer 1987); white poplar: 1% cellulase and 0.1% pectolyase, 1.5 h (Qiao et al. 1998); camphor tree: 3% cellulase and 3% macerozyme, 12 h (Du and Bao 2005); and American elm: 0.2% cellulase, 0.1% driselase, and 0.03% pectolyase for 2 h (Jones et al. 2015). Although the extension of the digestion period gradually increased the release of silk tree protoplasts, the mean viability of isolated protoplasts decreased considerably with only 54% after 12 h for leaves and 52% after 20 h for callus (Tables 1, 2). Similar detrimental effects of increased digestion period on viability of isolated protoplasts from different explants has been reported for other woody plants such as *Quercus acutissima* (sawtooth oak) and black locust (Wakita 1997; Wakita et al. 1992; Kanwar et al. 2009). Kanwar et al. (2009) reported a decrease in the viability of black locust callus-derived protoplasts with an increase in digestion period from 24 h (95.5% viability) to 28 h (63.2% viability). These results demonstrate that optimization of specific enzyme combinations and digestion period is necessary for any species and cell type.

The use of an osmoticum prior to enzyme digestion or included in the enzyme digestion solution to increase protoplast yield and viability has been well documented. Davey et al. (2005b) suggested that plasmolysis of source material in, for example, a solution containing mannitol or sorbitol prior to enzymatic digestion improved viability and reduced damage and spontaneous fusion of adjacent protoplast. Our results indicated that protoplast isolation based on plasmolysis of source tissue with an osmoticum was suitable for releasing a high yield of healthy protoplasts from leaves and callus tissue of *A. julibrissin*. Sixty- or 90-min-plasmolysis of leaves or callus tissue, respectively, in 0.7 M sorbitol was the most efficient pretreatment for recovering a high yield of viable protoplasts in silk tree (Table 3). Umate et al. (2005) used 0.5 M mannitol in the enzyme solution when isolating protoplasts from leaf tissue of mulberry. Pre-plasmolysis of European field elm leaf strips for 1 h in CPW salts with 0.7 M mannitol and 5 mM MES followed by enzyme digestion was reported by Conde and Santos (2006). In black locust, Kanwar et al. (2009) dissolved different concentrations of cellulase and macerozyme in 0.7 M mannitol. Guo et al. (2012) digested leaves of a *Populus* clone in an enzyme solution that included 0.4 M mannitol, 3% cellulase and 0.8% macerozyme for 5 h.

Cell wall formation, mitosis reactivation, and sustained cell divisions are key steps in the formation of microcolonies, callus formation, and subsequent plant regeneration from protoplast cultures (Eeckhaut et al. 2013). Improvement in protoplast-to-plant protocols have been accomplished by testing various culture methods and medium formulations (Davey et al. 2005a). Since the first report on regeneration of whole plants from protoplast-derived cells of tobacco (Nagata and Takebe 1971), numerous methods have been developed for a variety of plant species (Davey et al. 2005a, b; Eeckhaut et al. 2013). Plant protoplasts are generally cultured at an initial plating density of 5×10^4–1×10^6 Pp ml^{-1} (Davey et al. 2005b). In the present study, leaf- and calli- derived protoplasts cultured with a plating density of 3–3.5×10^5 Pp ml^{-1}, successfully formed cell walls and then microcolonies when cultured in both liquid and agarose culture medium. A higher rate of colony formation was recorded for protoplasts cultured in KM8p medium supplemented with 2.7 or 5.4 µM NAA plus 2.2 µM BA (Supplementary Table 2). Nutritional requirements of protoplasts varied according to species and different tissues of the same species from which protoplasts were isolated (Davey et al. 2005b). The basal media MS, B5, KM and other modified formulations have been successfully used to culture protoplasts of a variety of woody species (Conde and Santos 2006; Cai and Kang 2014; Jones et al. 2015; Rezazadeh and Niedz 2015). Different culture systems (liquid or agarose embedded) and PGRs have been successfully applied for protoplast culture of hardwood species (Kanwar et al. 2009; Jones et al. 2015). In black locust, maximum dividing cells from leaf mesophyll- and callus-derived protoplasts occurred when cultured in Nagata and Takebe (1971) medium and in WPM (without NH_4NO_3), respectively, containing 5 µM NAA plus 1 µM BA. In American elm, protoplasts initiated and continued cell division when embedded in agarose beads (37.5%) versus using liquid KM5/5 and alginate beads (Jones et al. 2015). In silk tree, leaf- and callus-derived protoplasts were more successful in microcolony formation during liquid culture than in the agarose KM8p embedded system.

Auxin and cytokinin are two of the main PGRs controlling plant cell division. In silk tree, microcolonies derived from leaf- and callus-derived protoplasts formed microcalli (2–5 mm in diameter) followed by proliferation in semi-solid MSB5 medium (MS minerals with Gamborg B5 organics) or WPM containing different concentrations of NAA in combination with 4.4 µM BA (Supplementary Table 3). Higher callus proliferation efficiency was observed from MSB5 medium with 10.8 µM NAA plus 4.4 µM BA for leaf-protoplasts, and from MSB5 medium with 8.1 and 10.8 µM NAA plus 4.4 µM BA, and WPM with 10.8 µM NAA plus 4.4 µM BA for callus-protoplasts. Similarly, the key role of NAA and BA in callus formation from protoplast culture of other

woody plants has been reported by Trémouillaux-Guiller et al. (1996) in *Ginkgo biloba* (Maidenhair tree), Marchant et al. (1997) in *Rosa hybrid* (rose), and Ortin-Parraga and Burgos (2003) in *Prunus armeniaca* (apricot).

Shoot regeneration of silk tree from leaf- and callus-protoplast derived calli based on our previous protocol (MS medium with 13.2 µM BA plus 4.6 µM zeatin; Rahmani et al. 2015) was satisfactory. The use of various PGRs and combinations for shoot regeneration from protoplast derived callus varies depending upon the species. In protoplast culture of hybrid poplar, different concentrations of zeatin were found more effective in shoot regeneration than BA and 6-(c,c-dimethylallylamino) purine (Park and Son 1992). Qiao et al. (1998) reported that poplar buds were induced on MS medium with 10 µM kinetin or 1 µM thidiazuron (TDZ). Mulberry shoots were regenerated from microcalli cultured on MS medium with 4.5 µM TDZ and 17.1 µM indole-3-acetic acid (Umate et al. 2005). Black locust leaf mesophyll- and callus-derived protoplasts produced shoots when cultured on MS medium with 0.5 µM NAA and 1 µM BA (Kanwar et al. 2009). American elm shoots were regenerated on MS medium with 10 or 20 µM TDZ (Jones et al. 2015). As with shoot regeneration, adventitious rooting of shoots depends upon the species. For silk tree, successful rooting was achieved using half-strength MS medium supplemented with 4.9 µM IBA. In vitro rooting experiments carried out with *Phellodendron amurense* (Azad et al. 2006), *Robinia pseudoacacia* (Kanwar et al. 2009), and *Ulmus americana* (Jones et al. 2015) all reported successful root induction on protoplast-derived shoots using various concentrations of IBA.

Genetic fidelity of in vitro regenerated plants is one of the most important prerequisites for regenerating tissue culture plants, because of the potential effect of culture conditions on somaclonal variation of regenerated plants (Mikula et al. 2011). In this work, the genetic fidelity of leaf- and callus-protoplast based plants was analyzed using two DNA-based molecular markers (SCoT and ISSR). SCoT markers have been developed based on the short conserved region flanking the ATG start codon in plant genes (Collard and Mackill 2009) and are generally informative, cost-effective, and reproducible for evaluation of the genetic stability of plants (Gorji et al. 2011; Rathore et al. 2014; Rahmani et al. 2015). The application of ISSR markers is a quick and cost-effective method based on PCR to amplify inter-microsatellite sequences multiple loci in the genome (Prevost and Wilkinson 1999; Essadki et al. 2006). The amplification profiles of all applied SCoT and ISSR primers were found monomorphic across all of the silk tree plants, and therefore demonstrated the genetic fidelity of leaf- and callus-protoplast-derived in vitro plants of *A. julibrissin*. Use of more than one DNA marker technique has been recommended to be advantageous for analysis of genetic fidelity, because each marker will reveal the homogeneity of different regions of the genome (Palombi and Damiano 2002; Martins et al. 2004). DNA-based methods such as ISSR, random amplified polymorphic DNA, SCoT, or inter-retrotransposon amplified polymorphism analysis have been used to validate the genetic homogeneity of in vitro culture plants (Palombi and Damiano 2002; Campbell et al. 2011; Rawat et al. 2013; Agarwal et al. 2015; Rahmani et al. 2015). Considering the importance of protoplast isolation and plant regeneration in biotechnology studies of woody plants; maintaining genetic stability of in vitro regenerated plants will be useful in somatic hybridization, cell structure and function studies, genetic improvement, and production of secondary metabolites of the important woody legume *A. julibrissin*.

Conclusion

A successful protocol for protoplast isolation and subsequent plant regeneration from in vitro leaves and hypocotyl-derived callus was developed for *A. julibrissin*. The high-yielding protoplast tissue, effective period for digestion of tissues, preparation of source tissues, and appropriate concentrations and combinations of enzymes in the digestion solution were defined for isolation of maximum viable silk tree protoplasts. Protoplast callus induction and subsequent plant regeneration were demonstrated, showing that *A. julibrissin* was amenable to protoplast regeneration. Therefore, the protocol described here will enable us to apply technologies that require protoplasts; in genetic manipulation, studying plant physiological responses through cell-based experiments involving gene expression regulation, signal transduction, and protein targeting of this ecologically, economically, and medicinally beneficial tree legume.

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